

# Molecular Analysis and Effectiveness Assay of AVI Gene in Transgenic Tobacco for Resistance to *Begomovirus*

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## ABSTRAK

**Analisis Molekuler dan Uji Keefektifan Gen AVI pada Tanaman Tembakau Transgenik untuk Ketahanan terhadap Infeksi *Begomovirus*. Tri J. Santoso, Muhammad Herman, Sri H. Hidayat, Hajrial Aswidinnoor, dan Sudarsono.** Transformasi genetik tanaman tembakau dengan gen AVI *Begomovirus* telah dilakukan pada penelitian sebelumnya dan telah menghasilkan tanaman tembakau transgenik putatif yang membawa gen ketahanan terhadap antibiotik kanamisin. Tujuan penelitian ini adalah untuk (1) memperoleh tanaman tembakau transgenik generasi T<sub>0</sub> yang membawa gen AVI *Begomovirus* berdasarkan teknik PCR, (2) mendapatkan informasi integrasi dan jumlah kopi gen AVI pada genom tanaman tembakau transgenik generasi T<sub>0</sub> dengan teknik Southern Blot dan korelasinya dengan respon ketahanan, (3) memperoleh tanaman tembakau transgenik generasi T<sub>0</sub> yang mempunyai ketahanan terhadap *Begomovirus*. Deteksi gen AVI dengan PCR pada tanaman tembakau transgenik dilakukan menggunakan primer spesifik untuk gen AVI-*Begomovirus*. Sedangkan untuk analisis Southern Blot dilakukan dengan menggunakan pelacak (*probe*) untuk gen AVI. Keefektifan gen AVI pada tanaman tembakau transgenik diuji dengan skrining menggunakan virus target yang diinokulasikan dengan vektor kutu kebul. Hasil penelitian menunjukkan bahwa terdapat korelasi positif antara keberadaan atau integrasi gen AVI *Begomovirus* pada tanaman tembakau transgenik dengan fenotipe ketahanan terhadap infeksi virus. Integrasi gen AVI yang bersifat kopi tunggal lebih tahan terhadap infeksi virus dibandingkan integrasi gen yang multikopi. Ketahanan yang diperoleh dari ekspresi gen AVI *Begomovirus* diindikasikan dengan tidak adanya gejala dan akumulasi virus pada jaringan tanaman. Analisis hibridisasi *Northern* atau *Western* perlu dilakukan untuk mengetahui ada tidaknya akumulasi mRNA atau protein, sehingga mekanisme ketahanan yang terjadi dapat dijelaskan lebih detail.

**Kata kunci:** Tembakau (*Nicotiana tabacum*), analisis molekuler, teknik PCR, Southern Blot, gen AVI-*Begomovirus*, transgenik.

## ABSTRACT

**Molecular Analysis and Effectiveness Assay of AVI Gene in Transgenic Tobacco for Resistance to *Begomovirus*. Tri J. Santoso, Muhammad Herman, Sri H. Hidayat, Hajrial Aswidinnoor, and Sudarsono.** Genetic transformation of tobacco plant using AVI gene was conducted at the previously experiment and generated transgenic tobacco plants positively carrying the selectable marker *nptII* gene. The objectives of this experiment were to (1) analyze the presence of *Begomovirus* AVI gene in T<sub>0</sub> generation putative transgenic tobacco plants using PCR technique with specific primers and its correlation with resistance phenotype, (2) analyze the integration and copy number of the transgene in T<sub>0</sub> generation putative transgenic tobacco plants and its correlation with resistance response, (3) screen the T<sub>0</sub> generation putative transgenic tobacco plants with the target virus infection and to detect the presence of the virus in the transgenic plant tissue using universal primers. PCR detection of AVI gene in tobacco transgenic was conducted by using specific primer for *Begomovirus* AVI gene. Meanwhile, Southern Blot analysis was conducted by using the AVI gene probe. The effectiveness of AVI gene in tobacco transgenic was tested by inoculation of target virus using whiteflies vector. Result of the experiments showed that there was a positive correlation between the presence of the AVI transgene in T<sub>0</sub> generation putative transgenic tobacco plants and the resistant phenotype. Transgenic plants with a single copy integration of the transgene exhibited more resistant than the multiple copy one, and non transgenic plant. The resistance as a result of AVI gene expression was indicated with no symptom in T<sub>0</sub> generation transgenic tobacco plants and the accumulation of the virus in the transgenic plants tissue. Northern and Western hybridization analysis need to be performed for investigating the presence of mRNA or protein accumulation so that the resistance mechanism of the AVI gene could be explained more detail.

**Keywords:** Tobacco (*Nicotiana tabacum*), molecular analysis, PCR technique, Southern Blot analysis, *Begomovirus* AVI gene, transgenic.

## INTRODUCTION

Recently, leaf curl disease associated with *Begomovirus* infection has become a serious threat in some vegetables crops such as tomatoes and peppers. The disease has reportedly spread across many areas of the two commodities indicated by the identification of *Begomovirus* in these areas (Aidawati *et al.*, 2005; Hidayat *et al.*, 2006; Santoso *et al.*, 2008). Currently, the available leaf curl disease control techniques are not effective to stop the spread of the disease. In addition, because of costly, the existing techniques are sometimes not eco-friendly either to the environment and human. The use of resistant varieties is a one of controlling techniques that is relatively cheaper and safer for the environment. However, sources of disease resistance gene to curl disease has not been found in both tomato and pepper germplasm. Based on this fact, biotechnology approaches can assist in the curl disease resistant plant breeding program.

There are two main approaches of the development of genetic resistance to the virus that depends on the source of genes used (Dasgupta *et al.*, 2003). Resistance genes can be derived from the virus itself or from other sources. The first approach is based on the concept of resistance derived from pathogens (pathogens-derived resistance, PDR). In this approach, a part of the gene or intact gene of the virus is introduced into the plant, which will further affect one or several important stages in the viral life cycle. Utilization of coat protein gene is one example of this PDR approach (Vidya *et al.*, 2000; Sinisterra *et al.*, 1999; Raj *et al.*, 2005). The second approach is the resistance that comes not from the pathogen (non-pathogen-derived resistance), but based on the utilization of the resistance genes of host plants and other genes responsible for adaptation and host plants response to pathogen attack. The use of non-PDR type of resistance, although not as popular as the PDR approach, has given great hope to develop a durable resistance.

In the study of genetic engineering to produce transgenic plants usually involves several stages in the molecular or cellular biology techniques, one of which is the characterization or identification of genes that have been introduced into plant tissues (Bennett, 1993). The success of genetic transformation techniques marked by the success of inserting the gene that introduced into the plant genome, can be expressed and maintained throughout subsequent cell division process. Therefore, efforts are needed to confirm the integrity of the introduced genes and determine the number of gene copies in the genomes

of plants and identify the gene can function correctly. Identification of the transformed plant tissue can be done with a number of molecular techniques including the use of *polymerase chain reaction* technique (PCR) (Chee *et al.*, 1991; Nain *et al.*, 2005) and Southern Blot (Chee *et al.*, 1991). PCR is a rapid detection method to determine the presence of transgene in putative transgenic plant tissues. In addition, Southern Blot technique is also a technique that can be used to detect transgene integration and the copy number of transgene. Several studies of genetic engineering to obtain virus-resistant transgenic plants always involves the use of molecular techniques such as PCR and Southern Blot to detect the introduced transgene (Pascal *et al.*, 1993; Raj *et al.*, 2005).

In previous experiments, genetic transformation of tobacco plants with *Begomovirus-AV1* gene through *Agrobacterium tumefaciens* vector has produced about 1399 transformant plants (Santoso *et al.*, 2011). Transgenic tobacco plants has brought kanamycin antibiotic resistance gene (*nptII*). However, the molecular analysis to detect the presence of the *AV1* genes on putative transgenic tobacco plants and test the effectiveness of the gene for resistance to *Begomovirus* have not done yet. Therefore it is necessary to test the effectiveness and molecular analysis of the *AV1* gene against *Begomovirus* from such putative transgenic tobacco plants.

The objectives of this study were (1) to analyze the integration of *AV1* gene in  $T_0$  generation putative transgenic tobacco plants using the PCR technique, (2) to determine the copy number of the transgene integrated into the genome of  $T_0$  transgenic tobacco, (3) to test the  $T_0$  transgenic tobacco plants that carry the *AV1* gene with *Begomovirus* infection.

## MATERIALS AND METHOD

### Isolation of Total Genomic DNA Putative Transgenic Tobacco Plants

$T_0$  generation of putative transgenic tobacco plants (46 plants) were used in this experiment. Isolation of total genomic DNA of  $T_0$  putative transgenic tobacco plants was conducted using a method developed by Doyle and Doyle (1990) with modification by the addition of 2% polyvinyl pyrrolidone (PVP). A total of 3 g of plant leaves were grinded and added with 700  $\mu$ l of extraction buffer (20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, 2% PVP, and 0.2% Mercaptoetanol) and incubated for 15 minutes at 65°C in water bath with occasional mixing

to avoid aggregation of the homogenate. To the extract then was added with 700  $\mu$ l of solution of phenol-chloroform-isoamylalcohol (25 : 24 : 1) (v/v/v) and the mixture was vortexed thoroughly. The suspension was centrifuged for 15 minutes at 10.000 x g. The debris-free supernatant was transferred to a new tube and the DNA was precipitated by adding 2.5 x volume of absolute ethanol and washed twice with 70% ethanol (v/v). The DNA pellet was dried and resuspended in 100  $\mu$ l of sterile distilled water. This DNA extract was stored at -20°C for further use.

#### **Amplification of AV1 Gene of Putative Transgenic T<sub>0</sub> Tobacco Plants**

Amplification of AV1 gene in the genome of putative transgenic T<sub>0</sub> tobacco plants was performed using specific primers. The PCR reactions were consisted of 2.0  $\mu$ l of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1.2  $\mu$ l - 50 mM MgCl<sub>2</sub>, 0.4  $\mu$ l - 10 mM dNTP mix, 1  $\mu$ l - 10 uM of each primer, 1 unit of Taq DNA polymerase, and 2  $\mu$ l of 10<sup>-1</sup> diluted total nucleic acid template. Double distilled water was added up to a total volume of 20  $\mu$ l. Amplification was conducted in an MJ Research PTC100 thermal cycler. Amplification profiles were consisted of one cycle of denaturation at 94°C for five minutes, 30 cycles of denaturation at 94°C for one minute, primer annealing at 55°C for one minute, and primer extension at 72°C for two minutes, and one cycle of final primer extension at 72°C for five minutes. The amplified PCR product was analyzed using agarose gel (1%) electrophoresis in 0.5x TBE buffer. The gel was stained with ethidium bromide and visualized under UV light using Chemidoc gel system (Biorad).

#### **Southern Blot Analysis**

Southern Blot analysis was carried out according to the procedures of Panaud *et al.* (1993). Total genomic DNA was isolated from transgenic plants, cut with restriction enzyme *Hind*III and then separated in an agarose gel and transferred to Hybond N<sup>+</sup> nylon membrane. Hybridization process was performed by using a AV1 gene probe that has been labeled with a non radioactive dig-11-UTP. The hybridized membrane was then visualized with NBT-BCIP staining.

#### **Bioassay of Transgenic Plants with *Begomovirus* Infection**

Generation T<sub>0</sub> of putative transgenic tobacco plants were grown in plastic-bag and transferred to insect-proof cages for *Begomovirus* infection. They were challenged with *Begomovirus* using viruliferous whiteflies (*Bemisia tabaci*) and maintained in an

insect-proof cage for 3-7 days in order to the vector can transmit the virus to the plants. After that, the tobacco plants were transferred to the greenhouse and the development of symptoms was observed at 2 weeks post-inoculation.

Observation of symptoms of tobacco plants infected by the *Begomovirus* was conducted by category: (-) not infected, no symptoms appear, and (+) infected, symptoms appear on plants that indicated by the presence of mosaic or curling leaf like a cracker. To confirm the existence of *Begomovirus* in the infected tobacco plants after bioassay, PCR analysis using universal primer was performed. Isolation of total DNA of virus-infected plants was carried out by using a method as described by Doyle and Doyle (1990). PCR amplification to detect the presence of the virus was carried out according to the procedure of Rojas *et al.* (1993). PCR was performed in a total of 25  $\mu$ l reaction mixtures containing 2-5  $\mu$ l of genomic DNA template and a pair of universal primer PAL1v1978 and PAR1c715. The presence of the virus in transgenic tobacco plant tissues after bio-assay is indicated by 1.500 bps DNA fragments.

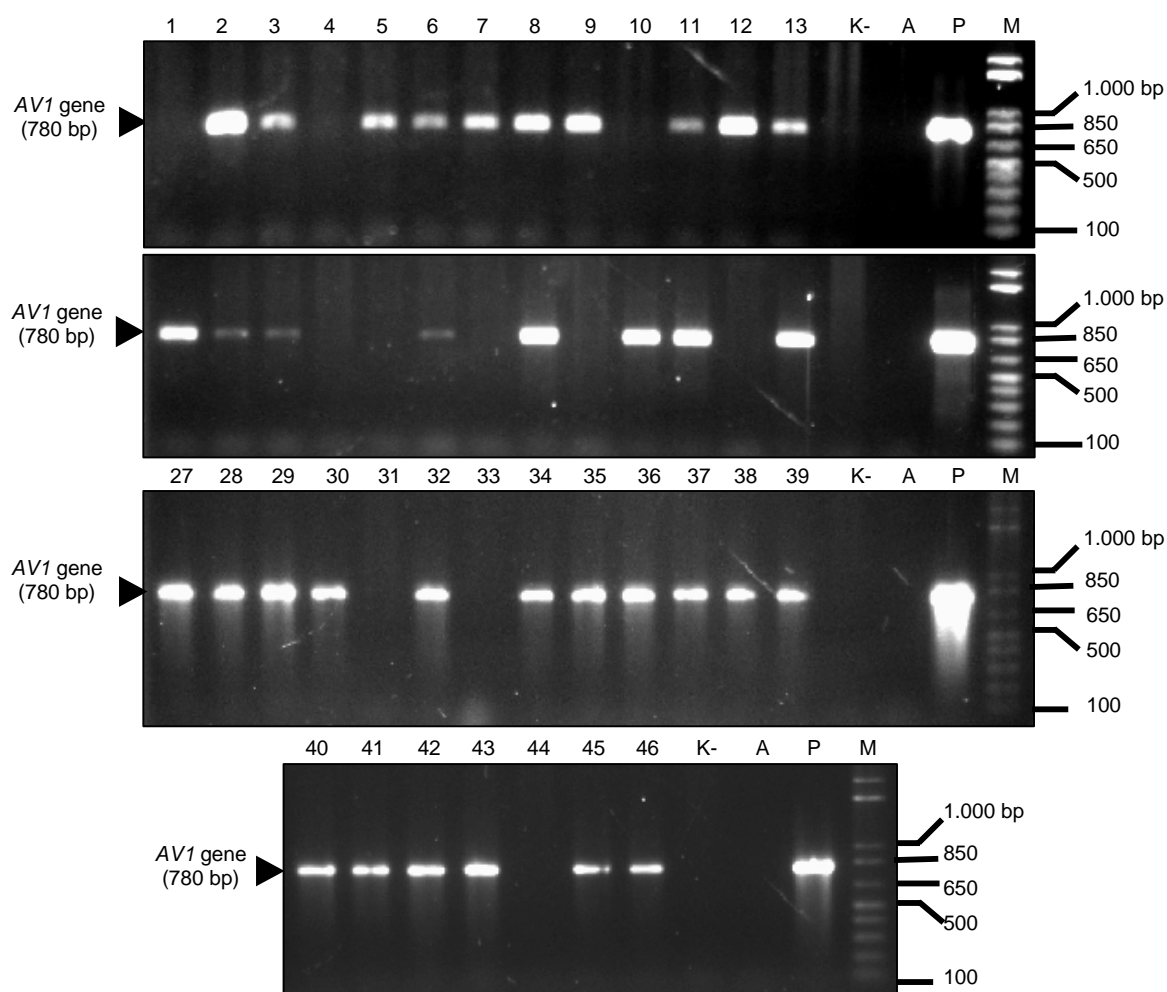
### **RESULT AND DISCUSSION**

#### **PCR Analysis to Detect the AV1 Gene of T<sub>0</sub> Generation Plant**

PCR analysis using the AV1 gene-specific primer was performed in order to detect the presence of the desired gene in a transformed plant tissue. PCR analysis can also be used for rapid screening of transformed plants carrying the transgene. The results of PCR analysis of the 46 putative transgenic tobacco plants of T<sub>0</sub> generation showed that there were 35 plants gave an expected PCR amplicon of 780 bps (Figure 1) but no such amplicon was observed in negative controls (non-transformed tobacco plants and water). The percentage of the PCR positive plants (containing AV1 gene) were 76.1%.

#### **Bioassay of the Putative Transgenic Plant with *Begomovirus***

The T<sub>0</sub>-generation transgenic tobacco plants, when were challenged by *Begomovirus* through viruliferous whiteflies in the insect-proof cage showed varies of response against *Begomovirus* compared to the non-transformed control plants (Figure 2). Out of 46 plants only 15 putative transgenic tobacco plants (32.6%) showed symptoms of virus infection and there were two plants that died before observation. Indications of tobacco plants infected by *Begomovirus* are the emergence of symptoms beginning with the



**Figure 1.** PCR analysis of 46 putative transgenic tobacco plants of  $T_0$  generation using a pair of specific primers. 1-46 = samples of putative transgenic tobacco plants of  $T_0$  generation, K- = negative control tobacco plants, A = water, P = plasmid, M = 1 Kb plus ladder (Invitrogen).

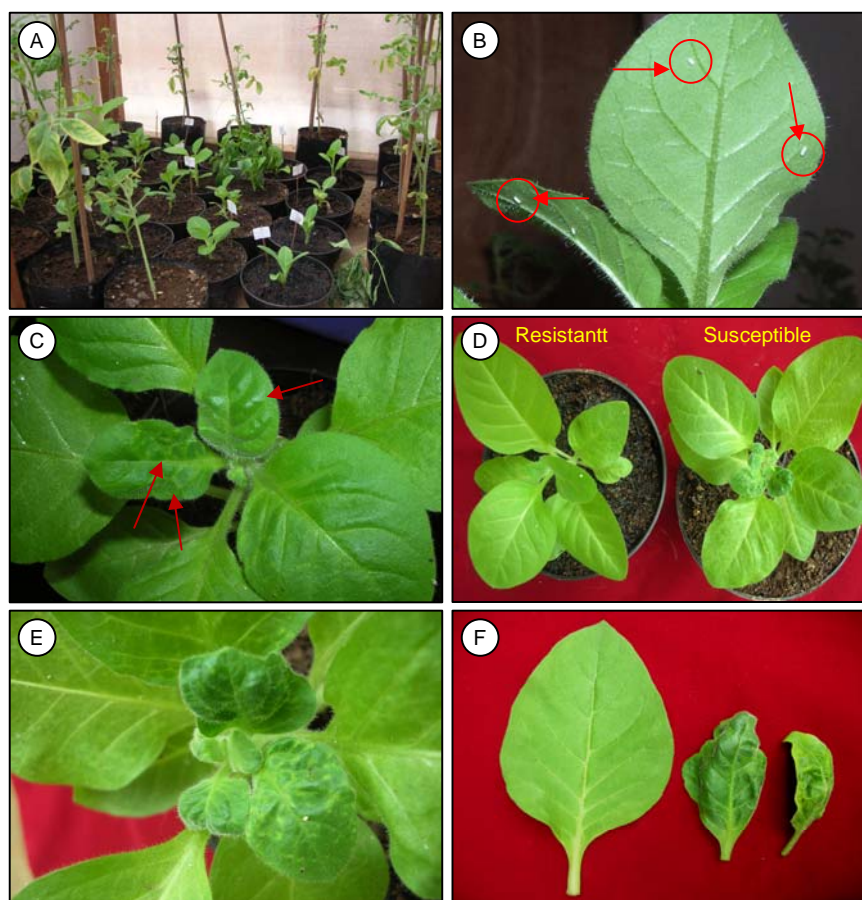
mosaic on the leaves and subsequent leaf blade will curl, wavy irregular like a cracker shape (Figure 2c-f). Based on PCR analysis and bioassay, it could be determined a correlation between the results of PCR analysis and bioassay of each individual putative transgenic tobacco plant (Table 1). There was a tendency that the PCR positive tobacco plants showed no symptoms after infection by the virus.

Based on the results of PCR analysis and bioassay, the putative transgenic tobacco plants  $T_0$  generation can be grouped into four categories (Table 2).

#### **Southern Blot Analysis and Confirmation of Existence the Virus**

Southern Blot technique using probe of *AV1* gene was used to determine the copy number of the

transgene that integrated into the genome of transgenic tobacco plants. The results of Southern Blot analysis in transgenic tobacco plants showed that out of 11  $T_0$  generation putative transgenic plants tested, were obtained five plants showed a single copy gene integration and six plants have more than one copy or multiple copies gene integration (Figure 3). Five transgenic tobacco plants that have a single copy were the plants of No. 2, 24, 27, 32, and 35, while six plants with multiple copies integration were the plants of No. 21, 23, 26, 34, 45, and 46. Two of the transformed tobacco plants that showed negative PCR (plant No. 10 and 20) were also included in Southern Blot analysis. Both of these plants showed no DNA bands after hybridization, similarly with the two non-transformed (NT) plants. For the positive control (P) that is the plasmid containing *AV1* gene, generated DNA bands.



**Figure 2.** Bioassay of transgenic tobacco plants using whiteflies insect vectors: A = the process of virus transmission by whiteflies vectors in insect-proof cages, B = whiteflies attached to the leaves of plants to transmit the virus (arrows), C = the symptoms began to appear on young leaves after virus inoculation at 2 weeks after inoculation (arrows), D = appearance of plants resistant and susceptible tobacco after inoculation with the virus, E and F = symptoms of viral infection on tobacco leaves.

To detect the presence of *Begomovirus* in the transgenic tobacco plants, total DNA was isolated from leaf tissue of lines and amplified using the universal primer to generate the specific DNA bands (Figure 4). Virus was detected in six samples of tobacco plants (plant No. 21, 23, 26, 34, 35, and 45). In the controls, NT-I (non-transformed plants but infected by the virus) and K+ (plants that infected by *Begomovirus*) the virus were detected in plant tissues, while NT-NI (non-transformed plants but not infected by virus) were not detected.

By combining the results of PCR analysis, bioassay, Southern Blot and detection of virus the level of resistance of these transgenic tobacco plants can be determined (Table 3).

To study the expression of gene that encode a AV1 protein associated with resistance to leaf curl disease caused by infection of *Begomovirus*, the AV1 gene was introduced into the genome of tobacco

plants that act as a model plant. Information on the integration, copy number and expression of AV1 gene in transgenic model plants are expected to be useful for understanding the effectiveness of the gene to control curly leaf disease. Therefore, the gene can be used for developing of tomato or pepper varieties resistant to *Begomovirus*. The utilization of viral genes to obtain resistance properties have been conducted by several researchers, including the BL1 gene that encode bipartite *Begomovirus* of the movement protein (Pascal *et al.*, 1993), AV2 gene of *Begomovirus* monopartit that encode movement proteins (Mubin *et al.*, 2007), CP gene from Tobamovirus (Bendahmane *et al.*, 1997).

Kanamycin-selected plants were tested by PCR to identify that they carry the transgene using AV1 gene specific primers. Forty-six tobacco T<sub>0</sub> plants were tested by PCR and 35 of them were indeed positive transgenic indicated by DNA fragments sized of 780 bp

**Table 1.** Results of PCR analysis of AVI genes and bioassay of T<sub>0</sub> generation putative transgenic tobacco plants in the greenhouse.

No.	Sample code	PCR*	Bioassay**	No.	Sample code	PCR*	Bioassay**
1.	CP8/II.1.2.1	-	+	27.	CP8/II.1.3.1	+	-
2.	CP11/I.2.1.3	+	-	28.	CP8/II.1.3.2	+	-
3.	CP11/I.2.4.1.A	+	-	29.	CP8/II.2.1.1	+	+
4.	CP11/I.2.4.1.B	-	died***	30.	CP8/II.2.3.1	+	-
5.	CP11/I.3.2.1	+	-	31.	CP8/II.2.3.2	-	+
6.	CP11/I.4.2	+	+	32.	CP8/II.5.1.1	+	-
7.	CP11/I.4.2.2	+	-	33.	CP8/II.5.1.2	-	+
8.	CP11/I.4.3.1	+	-	34.	CP8/II.13.1.1	+	+
9.	CP11/I.4.3.2	+	-	35.	CP8/II.13.2.1	+	-
10.	CP8/II.1.1	-	+	36.	CP8/II.13.3.1	+	-
11.	CP8/II.1.3.1	+	-	37.	CP8/II.14.1.1	+	-
12.	CP8/II.4.1	+	-	38.	CP8/II.1.5.x	+	+
13.	CP8/II.4.1.2	+	died***	39.	CP8/III.2.3.1	+	-
14.	CP8/II.4.2	+	-	40.	CP8/III.3.1.1	+	-
15.	CP8/III.1.4.2	+	-	41.	CP8/III.3.1.2	+	-
16.	CP8/III.2.1.1	+	-	42.	CP8/III.3.3.1	+	-
17.	CP8/III.2.1.2	-	+	43.	CP8/III.3.3.2	+	-
18.	CP8/III.2.4.1	-	-	44.	CP8/III.11.1.1	-	-
19.	CP8/III.3.2.1	+	-	45.	CP11/IV.5.1.1	+	-
20.	CP8/III.7.1.1	-	+	46.	CP11/IV.9.1.1	+	-
21.	CP11/I.2.1.1	+	+	47.	Control 1	-	+
22.	CP11/I.2.1.2	-	+	48.	Control 2	-	+
23.	CP11/I.2.2.2	+	+	49.	Control 3	-	+
24.	CP11/I.3.4.1	+	-	50.	Control 4	-	+
25.	CP11/I.6.1.1	-	+	51.	Control 5	-	+
26.	CP11/I.6.3.1	+	+				

\* PCR: (+) = generated the 780 bp DNA fragment, (-) = absent of the 780 bp DNA fragment; \*\* Bioassay: (+) = showed *Begomovirus* symptoms, (-) = no symptom; \*\*\* died before observation.

**Table 2.** Grouping of categories of putative transgenic tobacco plants based on the PCR analysis and bioassay.

Category	PCR analysis*	Bioassay **	Number of plant
1	+	-	28
2	+	+	7
3	-	+	8
4	-	-	3
Kontrol	-	+	5

\* PCR results: (+) = shown the DNA fragments sized of 780 bp, (-) = not shown; \*\* Bioassay: (+) = shown the symptomatic infected *Begomovirus*, (-) = not shown.

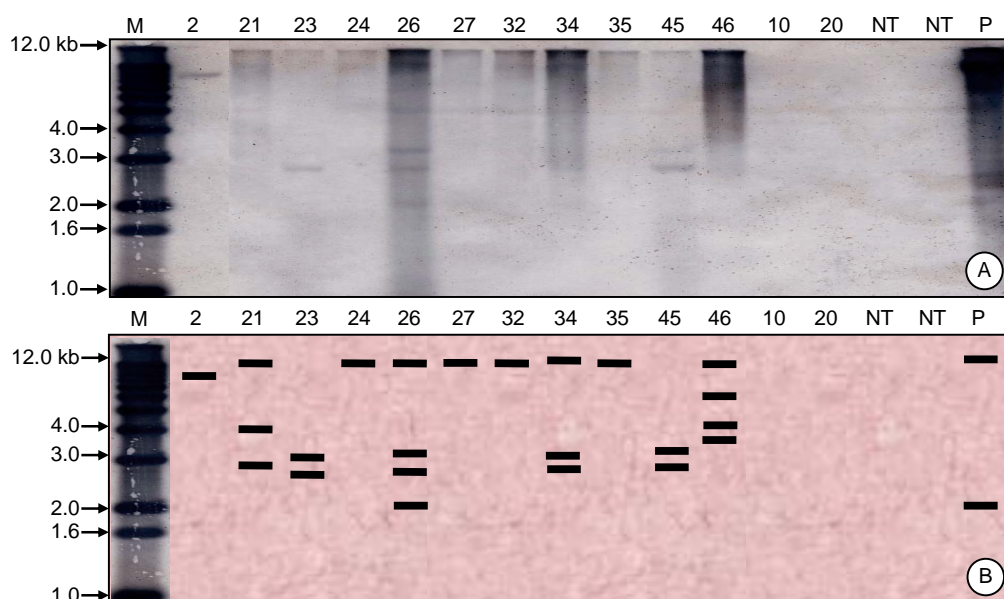
(Figure 4). It means that even though the all putative transgenic tobacco plants were resistant to kanamycin 100 mg/l in selection media, several of them (11 plants) did not contain the transgene.

Transgene expression in transgenic tobacco plants was assayed by virus infection. Most of transgenic tobacco plants that were PCR positive (carrying the AVI gene) showed resistant to virus infection compared to non-transgenic plants. Out of 35 plants with PCR positive, 28 plants showed no symptoms after the virus infection (Table 2). Seven plants with PCR positive which indicated the presence of symptoms thought to be caused by the ineffectiveness of AVI gene that has been integrated into the plant genome and is referred to as gene silencing process. In this study, the existence of plants

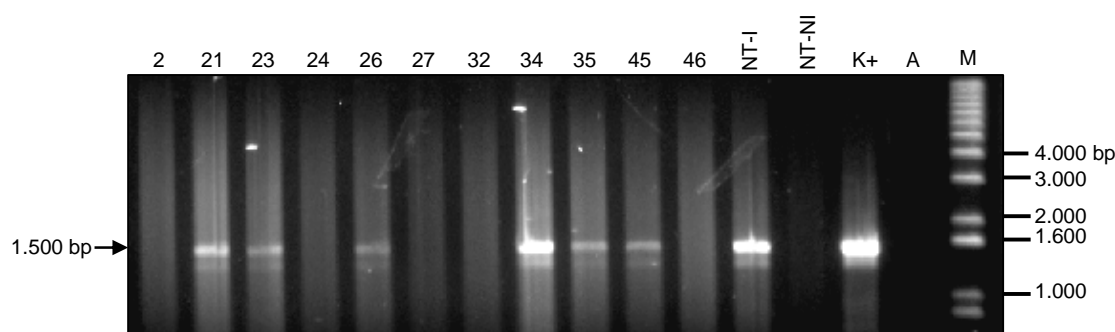
with PCR negative that showed no symptoms of virus infection was also observed. These results thought to be caused by non-occurrence of the virus transmission by whiteflies to the plants. One disadvantage of bioassay techniques in this study is the process of transmission of the virus depends on the mobility of the infestation of whiteflies vector, probably the process of viral infection by the insect was not occurred or escape.

Analysis of the copy number using Southern Blot technique indicated that the integration of AVI gene via *A. tumefaciens* into tobacco plants showed one copy or multiple copies (Figure 3). Correlation of the number of copies of AVI gene with the symptom occurrence indicated that the plants with the one copy gene integration did not show any symptoms after





**Figure 3.** Southern Blot hybridization analysis on samples of transgenic tobacco plants with a probe of AV1 gene. NT = non-transformed plants, P = plasmid and M = 1 Kb plus ladder. A = the original results of Southern Blot analysis, B = replica of Southern Blot analysis result.



**Figure 4.** Begomovirus detection by PCR using universal primer at T<sub>0</sub> generation transgenic tobacco plants after bioassay. Samples of plants is indicated by number. NT-I = non transformed plant, inoculated virus, NT-NI = non-transformed plant, non- inoculated, K+ = infected plant, A = water and M = 1 Kb ladder (Invitrogen).

virus infection. These result were supported by PCR data which no specific DNA fragments of the *Begomovirus* genome can be detected to identify the presence of virus in the plant tissues. Based on the data, these plants were categorized as resistant plants (Figure 4, Table 3). Meanwhile, plants with multi-copy integration of AV1 gene showed symptoms of viral infection and is supported by the detection of the presence of *Begomovirus* in the plant and the plants were categorized to susceptible plants (Figure 4, Table 3). The multiple copies integration was expected to induce gene silencing so that the transgene was not expressed and plants became susceptible and virus could not develop in the plant tissue. Gene silencing caused by the presence of the multiple copy of the transgene possibly related to the phenomenon of

homology-dependent gene silencing in which no expression of one or more genes because of the arrangement of nucleotide similarity (homology) (Meyer and Saedler, 1996). Interestingly, this phenomenon may not occur in transgenic plant No. 46 where the plant had four copies of genes but showed no revealing any symptoms of the virus in plant tissues. There might be other mechanisms of resistance caused the plants became resistant even though its integration is more than one. In this study, the transgenic plant with two copies of transgene (plant No. 45) and showed no symptoms but it exhibited viral accumulation in the plant tissues was also observed. This result was apparently related to a mechanism of tolerant response or recovery of the plant.

**Table 3.** Determining the respon of transgenic tobacco plant based on the result of PCR analysis, bioassay, Southern Blot and virus detection.

No.	Plant code	PCR	Bioassay	Copy number	Virus presence	Category
1.	2 (CP11/I.2.1.3)	+	-	1	-	Resistant
2.	21 (CP11/I.2.1.1)	+	+	3	+	Susceptible
3.	23 (CP11/I.2.2.2)	+	+	2	+	Susceptible
4.	24 (CP11/I.3.4.1)	+	-	1	-	Resistant
5.	26 (CP11/I.6.3.1)	+	+	4	+	Susceptible
6.	27 (CP8/II.1.3.1)	+	-	1	-	Resistant
7.	32 (CP8/II.5.1.1)	+	-	1	-	Resistant
8.	34 (CP8/II.13.1.1)	+	+	2	+	Susceptible
9.	35 (CP8/II.13.2.1)	+	-	1	+	Tolerant
10.	45 (CP11/IV.5.1.1)	+	-	2	+	Tolerant
11.	46 (CP11/IV.9.1.1)	+	-	4	-	Susceptible

In this study, it clearly showed that the transgenic tobacco plants having a single copy transgene were resistant to *Begomovirus*. These transgenic tobacco plants exhibited the absence of symptoms after virus infection and viral accumulation in the plant tissue. Based on the result, this resistance mechanism differs from the resistance based on RNA (RNA-mediated resistance) which the resistance is often associated with the presence of multiple copy integration of the transgene or a tandem repeat of the transgene. Based on the research data, mechanism of the resistance in this study was associated with the presence of *AVI* gene product. Once the *AVI* gene has integrated into the genome of the plant, the gene will be expressed and its protein product will affect the life cycle of the virus. In other words, the plant will express the *AVI* genes and accumulate of the coat proteins in plant tissue so when the virus infection occurs, the virus will be coated by coat proteins and the virus may not be able to develop in the plant tissue. This resistance mechanism has a role for the initial level of viral replication process by blocking the process of an uncontrolled replication of the virus particle (Aswidinnoor, 1995). However, the mechanism of this resistance remains to be proven further by Northern or Western hybridization analysis to study the presence or absence of mRNA or protein accumulation, so that the resistance mechanisms occurred can be described in more detail.

The viral coat protein genes has been used widely in genetic engineering techniques for the development of virus-resistant plants with RNA genome (Bendahmane *et al.*, 1997; Chowrira *et al.*, 1998; Srivastava and Raj, 2008). Research to acquire resistance to *Begomovirus* using coat protein genes has also been performed (Kunik *et al.*, 1994) and the results showed that TYLCV resistance associated with the presence of the transgene product. The resistance plant indicated the absence of symptom severity and

virus accumulation in plant tissues. In this study, the resistance of transgenic tobacco plants to *Begomovirus* indicated a phenomenon similar to experiment studies performed by Kunik *et al.* (1994).

### CONCLUSION

1. Transgenic tobacco plants carrying the *AVI* gene based on PCR amplification were obtained in the study.
2. Southern Blot hybridization analysis of the *AVI* gene integration into the genome of tobacco plants showed a single or multiple copies.
3. Plants with a single copy integration of genes showed a response resistant to virus infection compared to the integration of multiple-copy genes.
4. The resistance obtained from *Begomovirus AVI* gene expression in transgenic tobacco plants is indicated by the absence of symptoms and virus accumulation in plant tissues.

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